

Serial No.: 09/914,277
National Phase of: PCT/US00/04798
Attorney Docket No. 1059.00051


REMARKS

The above amendments add no new matter and are merely made to more accurately describe and claim the invention, to claim benefit of priority, and to eliminate multiple claim dependencies.

The Commissioner is authorized to charge any fee or credit any overpayment in connection with this communication to our Deposit Account No. 11-1449.

Respectfully solicited,

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CERTIFICATE OF MAILING

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Angel Webb

VERSION WITH MARKINGS TO SHOW CHANGES MADE

IN THE SPECIFICATION:

Page 40, Lines 5-16:

At first, the cDNA fragment, without adaptors encoding plasminogen kringles 1 to 5, was amplified by a sense primer and a complementary primer: 5'-CTCTCAGAGTGCAAGACTGGGAATGGAAAGAAC (Seq. ID. No. 1) (Leu100-Asn110), and 5'-GGCCGCACACTGAGGGACATCACAGTAGTC (Seq. ID. No. 2) (Ala562-Asp533 according to the amino acid sequence of human plasminogen) (Folkman, 1996). A cDNA fragment encoding collagen a1(XVIII) C-terminus was amplified by a set of primers: 5'-CACAGCCACCGCGACTTCCAGCCGGTGCTC (Seq. ID. No. 3) (His1154-Leu1163) for the sense 5'-end of the peptide, and 5'-CTACTTGGAGGCAGTCATGAAGCTGTTCTCAAT (Seq. ID. No. 4) (Lys1336-Ile1327) for the complementary 3'-end (Folkman, 1996). Amplification of cDNA fragments performed by using a proof-reading thermostable Pfu DNA polymerase (Stratagene, La Jolla, CA).

Page 40, Lines 24-31 and Page 41, Lines 41, Lines 1-9:

Addition of adaptor to the amplified cDNA fragments was performed by PCR using adaptor anchored primers. Primers for cloning of the human plasminogen kringle 5 were 5'-GGAATTCCATATGGAAGAAGACTGTATGTTTGGG (Seq. ID. No. 5) (G-[EcoRI]-[NdeI]-[Glu478-Gly486]), and 5'-GGAATTCCATATGGGCCGCACACTGAGGGACATC (Seq. ID. No. 6) (G-[EcoRI]-[NdeI]-[Ala562-Asp556]). Collagen a1(XVIII) C-terminus with adaptors were amplified using primers 5'-GGAATTCCATATGCACAGCCACCGCGACTTCCAG (Seq. ID. No. 7) (G-[EcoRI]-[NdeI]-[His1154-Ile1160]), and 5'-CCGGGATCCCTACTTGGAGGCAGTCATGAAGCT (Seq. ID. No. 8) (CCG-[BamHI]-[STOP]-[Lys1336-Ser1330]). PCR reaction solution (100 µl) contains 50 mM Tris (pH 8.8), 2 mM MgCl, 10 mM KCl, 10 µl of the above resulted PCR reaction mixture

containing the cDNA fragments, 250 ng of each primer and 7.5 units of Pfu DNA polymerase. Ten reaction PCR cycles consists of 45 sec at 94°C for denaturing, 45 sec at 37°C for annealing and 2 mins. At 68°C for DNA synthesis.

Page 53, Lines 4-17:

The tPA kringle 2 carrying mutation of His244→Tyr, is fused to collagen α1 (XVIII) C-terminus. A 558 bp DNA fragment encoding 183 amino acid residues of human collagen α1 (XVIII) C-terminus with adaptors that are amplified using primers 5'- GGAATTCCATATGCACAGCCACCGCGACTTCCAG (Seq. ID. No. 9) (G-[EcoRI]-[NdeI]-[His1154→Gln1160]), and 5'- CCGGGATCCCTACTTGGAGGCAGTCATGAAGCT (Seq. ID. No. 10) (CCG-[BamHI]-[STOP]-[Lys1336→Ser1330]). A 287 bp cDNA fragment encoding 87 residues of tPA kringle 2 mutant H are amplified by primers 5'- GGAATTCCATAACAGTGACTGCTACTTTGGG (Seq. ID. No. 11) (G-[EcoRI]-[NdeI]-[Asn177→Gly183]), and 5'- GGAATTCCATATGGGTGGAGCAGGAGGGGCACATC (Seq. ID. No. 12) (G-[EcoRI]-[NdeI]-[Thr263→Asp257]).

Page 80, Lines 26-31 and Page 81, Lines 1-6:

59°C for annealing and 3 minutes at 68°C for DNA synthesis. Kringle 1 was amplified by a 5'-end primer of GGAATTC-[NdeI]- ATAGATACCAGGGCCACGTGCTACG (Seq. ID. No. 13), and a 3'-end primer of CCG-[BamHI]-TTAGTTTCCCTCAGAGCAGGCAGG (Seq. ID. No. 14). Kringle 2 was amplified by a set of primers: GGAATTC-[NdeI]- AACAGTGACTGCTACTTTGGG (Seq. ID. No. 15) for 5'-end and CCG-[BamHI]- TTAGGTGGAGCAGGAGGGGCACATC (Seq. ID. No. 16) for 3'-end. A DNA fragment containing both of kringles was also amplified by a 5'-end primer of GGAATTC-[NdeI]-ATAGATACCAGGGCCACGTGCTACG (Seq. ID. No. 17), and a 3'-end primer CCG-[BamHI]-TTAGGTGGAGCAGGAGGGGCACATC (Seq. ID. No. 18). Where

GGAATTC-[NdeI]- and CCG[BamHI]- are adaptors containing NdeI and BamHI recognition sites.

Page 82, Lines 6-31:

Oligonucleotide primers designed for mutagenesis PCR are listed. Where, the mutation sites are underlined. Residue numbers are according to the amino acid sequence of tPA.

S mutant (Ser186 - Lys mutation):

SKf-1, sense, 5'-GCTACTTTGGGAATGGGAAAGCCTACCGTGGC-3' (Seq. ID. No. 19)

SKr-2, anti-sense, 5'-GCCACGGTAGGCTTTCCCATTCCCAAAGTAGC-3' (Seq. ID. No. 20)

Y mutant (Tyr214 - Phe mutation):

YFf-3, sense, 5'-CCTGATAGGCAAGGTTTTCACAGCACAGAACCCC-3' (Seq. ID. No. 21)

Yfr-4, anti-sense, 5'-GGGGTTCTGTGCTGTGAAAACCTTGCCTATCAGG-3' (Seq. ID. No. 22)

N mutant (Asn218 - Thr mutation):

NTf-5, sense, 5'-GTTTACACAGCACAGACCCCCAGTGCCCAGGC-3' (Seq. ID. No. 23)

NTr-6, anti-sense, 5'-GCCTGGGCACTGGGGGTCTGTGCTGTGTAAAC-3' (Seq. ID. No. 24)

G mutant (Gly225 - Glu mutation):

GEf-7, sense, 5'-GTGCCCAGGCACTGGAACTGGGCAAACATAAT-3' (Seq. ID. No. 25)

GEr-8, anti-sense, 5'-ATTATGTTTGCCCAGTTCCAGTGCCTGGGCAC-3' (Seq. ID. No. 26)

K mutant (Lys240 - Gly-Gly mutation):

KGGf-9, sense, 5'-CCTGATGGGGATGCCGGTGGCCCCCTGGTGCCACG-3' (Seq. ID. No. 27)

KGGf-10, anti-sense, 5'-CGTGGCACCAAGGGGCCACCGGCATCCCCATCAGG-3' (Seq. ID. No. 28)

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H mutant (His244 - Tyr mutation):

HYf-11, sense, 5'-GCCAAGCCCTGGTGCTATGTGCTGAAGAACCGC-3' (Seq. ID. No. 29)

HYr-12, anti-sense, 5'-GCGGTTCTTCAGCACATAGCACCAGGGCTTGGC-3' (Seq. ID. No. 30)

W mutant (Trp253-Glu254 - Tyr-Asp mutation):

WEYDf-13, sense, 5'-CCGCAGGCTGACGTATGATTACTGTGATGTGCCC (Seq. ID. No. 31)

WEYDr-14, anti-sense, 5'-GGGCACATCACAGTAATCATACGTCAGCCTGCGG (Seq. ID. No. 32)

Page 102, Lines 23-31 and Page 103, Lines 1-25:

1. Change Y18I to M. Oligonucleotide primers will be
5'-CAGTGA CTGCATGTTTGGGAATGGG-3' (Seq. ID. No. 33)
and 5'-CCCATTCCCAAACATGCAGTCACTA-3' (Seq. ID. No. 34).
2. Change T191 to K. Oligonucleotide primers will be
5'-CCTACCGTGGCAAACACAGCCTCACC-3' (Seq. ID. No. 35)
and 5'-GGTGAGGCTGTGTTTGCCACGGTAGG-3' (Seq. ID. No. 36).
3. Change S193 to A. Oligonucleotide primers will be
5'-CCGTGGCACGCACGCCCTCACCGAG-3' (Seq. ID. No. 37)
and 5'-CTCGGTGAGGGCGTGCGTGCCACGG-3' (Seq. ID. No. 38).
4. Change S206 to A. Oligonucleotide primers will be
5'-CCCGTGGAAATGCCATGATCCTGATAG-3' (Seq. ID. No. 39)
and 5'-CTATCAGGATCATGGCATTCCACGGG-3' (Seq. ID. No. 40).
5. Change D236 to P. Oligonucleotide primers will be
5'-GCCGGAATCCTCCGGGGGATGCC-3' (Seq. ID. No. 41)
and 5'-GGCATCCCCCGGAGGATTCCGGC -3' (Seq. ID. No. 42).
6. Change K240 to G. Oligonucleotide primers will be

5'-GATGGGGATGCCGGGCCCTGGTGCC-3' (Seq. ID. No. 43)
and 5'-GGCACCAGGGCCCCGGCATCCCCATC- 3' (Seq. ID. No. 44).

7. Change W253 to Y. Oligonucleotide primers will be
5'-CGCAGGCTGACGTACGAGTACTGTG-3' (Seq. ID. No. 45)
and 5'-CACAGTACTCGTACGTCAGCCTGCG- 3' (Seq. ID. No. 46).
8. Change E254 to D. Oligonucleotide primers will be
5'-GGCTGACGTGGGACTACTGTGATGTG-3' (Seq. ID. No. 47) and
5'-CACATCACAGTAGTCCCACGTCAGCC- 3' (Seq. ID. No. 48).
9. Change S262 to A. Oligonucleotide primers will be
5'-GTGCCCTCCTGCGCCACCTAAGGATCC-3' (Seq. ID. No. 49) and
5'-GGATCCTTAGGTGGCGCAGGAGGGCAC-3' (Seq. ID. No. 50).

Page 104, Lines 5-31 and Page 105, Lines 1-4:

1. Mutate F182 to H. Oligonucleotide primers will be
5'-GACTGCTACCACGGGAATGGGTCAG-3' (Seq. ID. No. 51) and
5'-CTGACCCATTCCCGTGGTAGCAGTC-3' (Seq. ID. No. 52).
2. Mutate A223 to N. Oligonucleotide primers will be
5'-CCCAGTGCCCAGAACCTGGGCCTGG-3' (Seq. ID. No. 53) and
5'-CCAGGCCCAGGTTCTGGGCACTGGG-3' (Seq. ID. No. 54).
3. Mutate W242 to T. Oligonucleotide primers will be
5'-GATGCCAAGCCCACCTGCCACGTGCTG-3' (Seq. ID. No. 55) and
5'-CAGCACGTGGCAGGTGGGCTTGGCATC-3' (Seq. ID. No. 56).
4. Mutate R249 to P. Oligonucleotide primers will be
5'-GTGCTGAAGAACCCAGGCTGACGTG-3' (Seq. ID. No. 57) and
5'-CACGTCAGCCTGGGGTTCTTCAGCAC -3' (Seq. ID. No. 58).

5. Mutate L251 to V. Oligonucleotide primers will be
5'-GAACCGCAGGGTGACGTGGGAGTAC-3' (Seq. ID. No. 59) and
5'-GTACTCCCACGTCACCCTGCGGTTC-3' (Seq. ID. No. 60).
6. Mutate D257 to N. Oligonucleotide primers will be
5'-GTGGGAGTACTGTAACGTGCCCTCC-3' (Seq. ID. No. 61) and
5'-GGAGGGGCAGTTACAGTACTCCAC- 3' (Seq. ID. No. 62).
7. Mutate V258 to L. Oligonucleotide primers will be
5'-GAGTACTGTGATCTGCCCTCCTGCTC-3'(Seq. ID. No. 63) and
5'-GAGCAGGAGGGCAGATCACAGTACTC- 3' (Seq. ID. No. 64).
8. Mutate P259 to K. Oligonucleotide primers will be
5'-GTACTGTGATGTGAAGTCCTGCTCC-3' (Seq. ID. No. 65) and
5'-GGAGCAGGACTTCACATCACAGTAC-3' (Seq. ID. No. 66).

Page 108; Lines 16-19:

Amino Acid presentation of tPA kringle 2, (K2_{tPA})

NSDCYFGNGSAYRGTHSLTESGASCLPWNSMILIGKYTAQNPSAQLGLGKHNYCRNPDGAKPWCHVLKNRRLTWEYCDVPSCST (Seq. ID. No. 67)

Amino Acid presentation of tPA kringle 2, (K2_{tPA})

NSDCYFGNGSAYRGTHSLTESGASCLPWNSMILIGKYTAQNPSAQLGLGKHNYCRNPDGAKPWCHVLKNRRLTWEYCDVPSCST (Seq. ID. No. 67)
* * * * *

Page 109; Lines 1-23:

NSDCYFGNGSAYRGTHSLTESGASCLPWNSMILIGKYTAQNPSAQLGLGKHNYCRNPDGAKPWCHVLKNRRLTWEYCDVPSCST (Seq. ID. No. 67)

Enzymatic fragmentation of K2_{tPA} at its two glutamyl bonds are performed with the GluV8 form of glutamyl endopeptidase 1, at optimal conditions to yield three peptides. The reaction is stopped with Cbz-Leu-Leu-Glu-CH₂Cl and the fragments separated by hplc.

Glutamyl endopeptidase -(*Staph. aureus* V-8 *Protease*); cleavage after E(Glu)

NSDCYFGNGSAYRGTHSLTESGASCLPWNSMILIGKYTAQNPSAQLGLGKHNYCRNPDGAKPWCHVLKNRRLTWEYCDVPSCST (Seq. ID. No. 67)

Prolyl oligopeptidase is used to cleave specifically the Pro-Xaa bonds in K2_{tPA}. Since this protease cleaves only small polypeptides, it is used in combination with CNBr and GluV8 to generate smaller fragments. However, if K2_{tPA} is susceptible to this post-proline cleaving enzyme a 27-residue, three 16-18 residue and two small (4-6 residue) peptide fragments spanning the K2_{tPA} domain can be obtained.

Post-Proline cleaving enzyme; cleavage after P (Pro)

NSDCYFGNGSAYRGTHSLTESGASCLPWNSMILIGKYTAQNPSAQLGLGKHNYCRNPDGAKPWCHVLKNRRLTWEYCDVPSCST (Seq. ID. No. 67)

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Please replace the Sequence Listing that follows the Abstract of this application and replace it with the attached Sequence Listing.